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D O C U M E N T A T I O N P 0500/5075

ESTABLISHMENT OF

AN IN VITRO DNA ADDUCT FORMATION ASSAY FOR

MUTAGENS REQUIRING METABOLIC ACTIVATION

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ABBREVIATIONS (a,b)
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ABT : American Board of Toxicology

AHM : aryl hydrocarbon monooxygenase (EC 1.14.14.2)

ATA : aurintricarboxylic acid

BA : 2,3-benzanthracene

B(a)P: benzo(a)pyrene

BC : team Biochemistry

bp : base-pairs

BPB : bromophenol blue

BPDE : 7-beta,8-alpha-dihydroxy-(9-alpha,10-alpha)-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene, anti-(+)-benzo(a)pyrene-7,8-diol-9,10-epoxide

BSA : bovine serum albumin

CCC : covalently closed circular

cpm : counts per minute

CPP : cyclophosphamide

dATP : deoxyadenosine triphosphate

DMBA : 7,12-dimethylbenzanthracene

DMNA : dimethylnitrosamine

DMS : dimethylsulfate

DNA : deoxyribonucleic acid

dpm : disintegrations per minute

DTT : dithiothreitol

EC : enzyme code

EDTA : ethylenediaminetetraacetic acid

Ex : x as exponent to the base 10, e. g. E2 = 10^2

G6P : glucose-6-phosphate

G6PDH: glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

.GT. : greater than

x g : centrifugal force in terms of the constant of gravitation (1 x g = 9.81 m/s2)

(a) in addition to those which are explained immediately on the same page

(b) Units are given in accordance with SI units (Système International d'Unités).

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ABBREVIATIONS (continued)
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HPLC : high performance liquid chromatography
i. p. : intraperitoneal
.LE. : less than or equal to
MEC : minimum effective concentration
N : number of individual values
NAD : nicotinamide adenine dinucleotide, oxidized form
NADH : nicotinamide adenine dinucleotide, reduced form
NADP : nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH : nicotinamide adenine dinucleotide phosphate, reduced form
OC : open circular
PAH : polycyclic aromatic hydrocarbon
RNA : ribonucleic acid
RNase A: ribonuclease A
rpm : revolutions per minute
RSD : relative standard deviation
RT : room temperature
SDS : sodium dodecylsulfate
SOP : standard operating procedure
SVT : sucrose-versene-Tris
TE : Tris-EDTA
Tris : tris(hydroxymethyl)aminomethane
tRNA : transfer ribonucleic acid
U : unit
UV : ultraviolet
XC : xylene cyanol

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1 SUMMARY

In a previous INBIFO study (P 0500/3060) 2 in vitro assays for the formation of deoxyribonucleic acid (DNA) adducts by mutagens (i. e. the conformation and the sequencing assay) were developed. The main limitation of the assays was the fact that only direct-acting mutagens could be investigated. Indirect-acting promutagens, which require metabolic activation by the cytochrome P450-mediated mixed-function oxidases located in the microsomes of the cellular endoplasmic reticulum, could not be investigated because rat liver microsome preparations were contaminated with DNA nucleases which interfered with the assays. The main purpose of the present study was to extend the DNA adduct assays to promutagens by modifying the method used for preparing microsomes in order to remove DNA nuclease contamination. In order to provide preliminary data for the validation of the assays, a number of indirect and direct model mutagens of different chemical classes and known reactivity were assayed. The test substances assayed for their direct genotoxic potential, that is without microsomal activation, were ACRIDINE, ACTINOMYCIN D, ANTI-(+)-BENZO(A)-PYRENE-7,8-DIOL-9,10-EPOXIDE (BPDE) and FORMALDEHYDE. Potential promutagens assayed with microsomal activation were the polycyclic aromatic hydrocarbons 2,3-BENZANTHRACENE (BA), BENZO(A)PYRENE (B(a)P), 7,12-DIMETHYLBENZANTHRACENE (DMBA) and PYRENE, also CYCLOPHOSPHAMIDE (CPP), DIMETHYLNITROSAMINE (DMNA) and CATECHOL.

A number of different methods for preparing microsomes were evaluated on the basis of DNA nuclease contamination and promutagen activating activity as determined by the specific aryl hydrocarbon monooxygenase (AHM) activity. Nuclease content had to be low enough not to interfere with the assays under standard conditions. Also ribonucleic acid (RNA) contamination was checked as RNA is also a substrate for genotoxic substances and therefore competes with DNA in the assays and it was desirable to reduce the

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RNA content of the microsomes. The best method to take these demands into account included differential centrifugation together with high salt extraction and osmotic shock treatment of the microsomes. This procedure resulted in a marked reduction in the amount of microsomal-associated RNA. The incubations of the test substances with plasmid DNA and purified microsomes were run in the presence of the divalent cation-chelating agent ethylene-diaminetetraacetic acid (EDTA) to inhibit residual nuclease activity. Under these conditions AHM activity was retained, most RNA removed and DNA nuclease sufficiently suppressed to prevent unspecific fragmentation of the target DNA during the assay incubation. This optimized procedure was used to assay the test substances in the present study.

In the conformation assay a positive result was recorded when an increase in the proportion of open circular (OC) DNA relative to that of covalently closed circular (CCC) DNA could be seen by eye. For the sequencing assay a positive result was recorded when specific bands could be seen above the background DNA. In both assays DMNA and the polycyclic aromatic hydrocarbons (PAH) BA, DMBA, B(a)P and pyrene were assayed with microsomal activation, and acridine, actinomycin D, BPDE and formaldehyde were assayed without microsomal activation. Catechol and CPP were assayed without and with microsomal activation.

In the conformation assay BPDE and formaldehyde (reproducibility limited) were positive without, B(a)P, DMBA, DMNA and pyrene with microsomal activation. Catechol and CPP were positive, both in the presence and absence of microsomes. Acridine, actinomycin D and BA were negative in the conformation assay. The minimum effective concentration (MEC) without heat posttreatment was between 0.001 (B(a)P and pyrene) and 10 millimoles/liter (CPP). Heat posttreatment lowered the MEC for catechol and CPP. When radioactively

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labeled B(a)P was assayed in the conformation assay with microsomal activation, to look for the distribution of radioactivity bound to the supercoiled and the OC form of pBR322, it was observed that a rather high proportion of radioactivity was bound to the supercoiled fraction indicating that a significant proportion of DNA molecules although attacked, were not transferred into the OC form. Although an estimation of the absolute amount of radioactive products bound to the total DNA (CCC plus OC plus background) was not made, these findings may limit the applicability of the conformation assay. On the other hand, incubation of plasmid DNA with radioactive B(a)P in the presence of microsomes clearly demonstrated that this compound requires microsomal metabolic activation before it can bind to DNA. The results for CPP, however, were contradictory, i. e., a concentration dependent increase of DNA adducts formed could not be observed with increasing amounts of microsomal protein. In addition, this compound was also found to exhibit genotoxic activity without microsomal activation. The results with radioactive DMNA were ambigous.

In the sequencing assay, using the plasmid pUR222 as target DNA, only BPDE and CPP yielded specific adducts. In addition, catechol and formaldehyde caused unspecific DNA breakdown.

To increase the sensitivity of the sequencing assay, a fragment of approx. 230 bp, the PAL10 fragment, was used as a DNA target. B(a)P, DMBA, pyrene, catechol, CPP and DMNA were investigated in the sequencing assay with the PAL10 fragment. All except CPP were negative. Adduct signals seen for CPP appeared much more intensive when compared to the results of experiments in which pUR222 was used as target DNA. This supported the expectation that decreasing the size of the target DNA increases the sensitivity of the assay.

The results showed that the conformation assay could be extended to promutagens and that it was sensitive enough to yield positive

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results for known genotoxic agents. Positive responses of the conformation assay were obtained at concentrations similar to those giving rise to reverse mutations in the Salmonella/microsome mutagenicity assay. The sensitivity of the sequencing assay, however, was limited, even when the PAL10 DNA fragment was used. The DNA adducts obtained with BPDE and CPP yielded interesting data on the positions and nature of DNA adduct formation.

These assays may be useful in investigating unknown substances and appear to be applicable to assaying complex mixtures such as cigarette condensates.

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Institut für biologische
Forschung GmbH

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2 RESPONSIBILITY

2.1 Project Management

Study Director: .....

Dr.rer.nat. S. Yeats
Biologist (Diplombiologe)

2.2 Contributing Team

Biochemistry: .....

Dr.rer.nat. R.-A. Walk
Biologist (Diplombiologe), Biochemist and Toxicologist (ABT)

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3 QUALITY ASSURANCE STATEMENT

The study was conducted according to the Good Laboratory Practice Regulations (a).

Inspections on this study were performed by the quality assurance unit on 4.Dec.85. All findings were immediately reported to the study director and to the general management.

The documentation accurately reflects the study carried out and the results obtained.

.....
Quality Assurance Manager
E. Römer
Biologist (Diplombiologe)

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(a) Federal Register (1978)

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4 INTRODUCTION

It is generally accepted that the initial step in chemical carcinogenesis is damage to DNA resulting from covalent adduct formation by a genotoxic substance or 1 of its metabolites (reviewed by Heidelberger, 1975, Grover, 1979, Sims, 1980). The extent of DNA adduct formation in vitro by test substances could therefore serve as an indication of the carcinogenic potential of the substances.

In a previous study (INBIFO P 0500/3060) 2 in vitro assays for directly acting mutagens was developed. These made use of the fact that modification of certain sites of DNA bases may lead to apurinic or apyrimidinic sites and strand breaks. The 1st, the conformation assay, consisted of examining by means of agarose gel electrophoresis the conversion as a result of exposure to genotoxic agents of CCC, or supercoiled, plasmid DNA to the OC, or nicked, or linear forms as a result of the introduction of single- or double-strand breaks respectively. The 2nd, the sequencing assay, used techniques analogous to the chemical DNA sequencing method developed by Maxam and Gilbert, 1980. After chemical treatment of radioactively end-labeled linear plasmid DNA, modified bases were displaced and the strand cleaved at the damaged site by a series of reactions. The products were separated on thin polyacrylamide gels, visualized by autoradiography and compared with a control sequence ladder of the DNA, revealing the extent and exact positioning of damage.

2 related E. coli plasmid cloning vectors were chosen for these assays. The well-known plasmid pBR322 (Bolivar et al., 1977), whose sequence of 4563 base pairs has been determined, was used for the conformation assay. A smaller derivative of this plasmid, pUR222 (Rüther et. al., 1981), was used for the sequencing assay.

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The latter plasmid, which is approx. 2700 base pairs in size, has the advantage that it can be labeled and sequenced without first separating the labeled products because the 2 used restriction sites (BamHI and EcoRI) are separated by only a few basepairs. Therefore, the small labeled product leaves the sequencing gel with the buffer front.

While these assays provided a simple means of assaying directly-acting genotoxic agents, it was desirable to extend the assays to promutagens, which require metabolic activation by the microsomal mixed-function oxidase system. Preliminary attempts to assay substances in the presence of microsomal fractions were unsuccessful due to endogenous DNA nuclease activity in the microsomes which nicked or degraded the DNA. The main aim of this study was to extend the assays to promutagens by purifying the microsomes away from nucleases and/or inhibiting residual DNA nucleases. Various buffers and salt conditions, and differential centrifugation were used to purify the microsomes. To abolish the abundant DNA nuclease activity from the microsomal preparations, 1st, the influence of aurintricarboxylic acid (ATA), a nonspecific enzyme inhibitor (Bina-Stein and Tritton, 1976), which was formerly thought to be a specific inhibitor of DNA binding proteins (Blumenthal and Landers, 1973), was assayed. This, however, was without success because the AHM activity was severely inhibited in parallel to the DNA nuclease activity. On the other hand, most DNA nucleases require divalent cations for activity, and the chelating agent EDTA was found to be most effective to reduce DNA nuclease activity without affecting AHM activity.

A 2nd aim of this study was the further validation of the 2 assays as screening methods for genotoxic potential. To this end a range of both direct-acting and pro-genotoxic agents were assayed. Substances assayed in the presence of microsomal protein were DMNA and the PAH BA, DMBA, B(a)P and pyrene. Acridine, actinomycin D, BPDE, which is the activated diol epoxide form of B(a)P, and formaldehyde were assayed directly. Catechol and CPP were assayed in either way. The chemical structures of the test

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substances and some of their properties are shown in TABLES 1 and 2.

PAH include a number of potent carcinogens which require activation by the cytochrome P-450 mixed-function oxygenase system contained in rat liver microsomes. They are widespread environmental pollutants of the atmosphere, water, and food-chain. PAH are formed predominantly through incomplete combustion and pyrolysis of fossil fuels and other organic materials and are also found in cigarette smoke (International Agency Report, 1983).

1 of the the best-studied PAH is B(a)P, whose carcinogenicity (reviewed by Heidelberger, 1975) and mutagenicity in Salmonella typhimurium (McCann et al., 1975) and in mammalian cells (Huberman and Sachs, 1976, Bradley et al., 1981) has been well-documented. B(a)P has been shown to cause DNA strand breaks as determined by the alkaline elution method (Sina et al., 1983). Metabolic activation of B(a)P results in the formation of a large number of derivatives including the putative ultimate carcinogenic metabolite, BPDE, which is an anti-7,8-diol-9,10-epoxide B(a)P derivative (reviewed by Gelboin, 1980). BPDE reacts with DNA to form a major adduct with the N-2 position of guanine and also minor adducts with the N-7 position of guanine and the N-6 position of adenine (Jeffrey et al., 1979, 1980, Osborne et al., 1981).

DMBA is the most potent carcinogen of the PAH group of substances. Its active metabolite, like that of B(a)P, also appears to be a diol epoxide, 1 or possibly both of the anti and syn isomers of the 3,4-diol-1,2-epoxide (reviewed by Sims and Grover, 1981). Like B(a)P, DMBA is mutagenic in Salmonella typhimurium (Mc Cann et al., 1975) and in mammalian cells (Huberman and Sachs, 1976, Bradley et al., 1981) and causes DNA strand breaks in rat hepatocytes (Sina et al., 1983).

Pyrene and BA, also called naphthacene, are nonmutagenic and noncarcinogenic PAH (McCann et al., 1975, El-Bayoumy and Hecht, 1986, Hermann, 1981). Pyrene was found neither to cause DNA

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strand breaks (Sina et al., 1983) nor to induce DNA repair in hepatocytes (Williams et al., 1982). However, it is a potent cocarcinogen when applied together with B(a)P on mouse skin (Rice et al., 1984) and with B(a)P, beta-propiolactone, and 60Co-gamma radiation in a transformation assay (Baturay and Kennedy, 1986). BA, while alone nonmutagenic, enhances the mutagenicity of B(a)P in low doses and inhibits its mutagenicity when added in higher doses (Hermann, 1981).

Catechol, a phenolic compound (1,2-dihydroxybenzene) is like B(a)P a component of cigarette smoke and has been shown to have cocarcinogenic (Van Duuren and Goldschmidt, 1976, Hecht et al., 1981) and comutagenic (Yoshida and Fukuhara, 1983) activity in conjunction with B(a)P. Catechol appears to block the detoxification pathway of B(a)P metabolism, thus increasing the amount of adduct-forming diol epoxide produced in the cell (Hoffmann et al., 1985) and enhancing the formation of anti-BPDE-DNA adducts (Melikian et al., 1986). Another explanation for the cocarcinogenic action of catechol could be interference with DNA repair of B(a)P lesions (Atcheson et al., 1982). However, the precise mode of its cocarcinogenic action of catechol remains obscure. Catechol also increases the incidence of oesophageal papillomas in rats treated with methyl-N-amyl-nitrosamine (Mirvish et al., 1985). The reactive metabolite is thought to be a semiquinone radical (Marks and Hecker, 1969). Catechol induces sister chromatid exchanges and delays cell division in human lymphocytes after treatment in culture (Morimoto and Wolff, 1980). It also causes DNA strand breaks in vivo and in vitro (Yamada et al., 1985). Catechol is therefore suspected to play an important role in the carcinogenicity of cigarette smoke.

CPP is used extensively as an antineoplastic agent for the treatment of various cancers and as an immunosuppressive agent (e. g.

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Lane, 1959, Haar et al., 1960). It has also been implicated in causing cancer (e. g. Sieber and Adamson, 1975, Hunstein, 1975) and is mutagenic in Salmonella typhimurium (McCann et al., 1975). CPP is activated by microsomal monooxygenases to yield the main metabolite phosphoramide mustard (Struck et al., 1971, Hales, 1981), which forms both a mono and a crosslinking adduct with the N-7 position of guanine (e. g. Kircher et al., 1979).

DMNA, a potent carcinogen and mutagen, is metabolized to an alkyl diazonium ion which alkylates DNA (Pegg, 1980). Approx. 80 percent of total alkylation is of the N-7 position of guanine (for review see Singer, 1979). However, all oxygen atoms are also attacked and the guanine-O^6-methyl adducts are considered to be of particular significance in the carcinogenesis of DMNA (Goth and Rajewsky, 1974).

Acridine is an intercalating compound and is not mutagenic (Brown et al., 1980, Ferguson et al., 1985).

Actinomycin D, an antibiotic which inhibits DNA-dependent RNA synthesis (Reich et al., 1962) is used as an antineoplastic agent (Bull, 1984). Actinomycin D is nonmutagenic in Salmonella typhimurium (Rinkus and Legator, 1979) and in V79 cells (Bradley et al., 1981). However, it is carcinogenic in the rat (IARC Monograph series) and has been shown by the alkaline elution method to cause strand breaks in rat hepatocytes (Sina et al., 1983).

Formaldehyde is an ubiquitous substance found in many occupational settings, various food products and other consumer products, petrol and diesel exhaust and cigarette smoke (Report of the Federal Panel on Formaldehyde, 1982, Wynder and Hoffmann, 1979, Schauenstein et al., 1978, Mansfield et al., 1977). Formaldehyde

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is mutagenic in various prokaryotic and lower eukaryotic systems (reviewed by Auerbach et al., 1977) and also in mammalian cells (Goldmacher and Thilly, 1983). It has been shown to be a weak promoter in C3H/10T1/2 cells (Frazelle et al., 1983) and to cause nasal passage cancers in rats (Swenberg et al., 1980, Kerns et al., 1983). In addition, formaldehyde induces sister chromatid exchanges, chromosomal aberrations and micronuclei in mouse and human cells (Kreiger and Garry, 1983, Levy et al., 1983, Natarajan et al., 1983), causes DNA single-strand breaks and DNA-protein crosslinks (Grafstrom et al., 1984) and also reacts with amino and sulfhydryl groups in cellular macromolecules (Singer and Grunberger, 1983, Auerbach et al., 1977).

To increase the sensitivity of the sequencing assay it was to be expected that a smaller target DNA would offer less potential target bases for attack by a genotoxic agent and thus increase the effect observed at a particular base. Using a DNA fragment, PAL10, of approx. 230 bp in size, B(a)P, DMBA, pyrene, catechol, CPP and DMNA were assayed in the sequencing assay.

As B(a)P, CPP and DMNA could be obtained radioactively labeled from commercial sources these substances were also assayed in this form in the conformation assay in the presence of microsomes in order to obtain more insight into the binding capacity of these compounds to DNA and protein. In addition, with this kind of experiments the number of DNA adducts formed can be compared with the compound's DNA-nicking properties.

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5 METHOD

5.1 Isolation of Microsomes

Principle:	differential centrifugation with osmotic shock treatment of microsomes to remove loosely-associated proteins and microsome-associated RNA
Time	
Sampling:	-
Determination:	-
Sample material and quantity:	liver of 1 Sprague Dawley rat, bodyweight .GT.150 g
Results expressed in:	-
Equipment:	homogenizer: Potter-Elvehjem, glass, with motor-driven teflon pestle, B. Braun Melsungen AG, D-3508 Melsungen centrifuges: RC-5B, rotor: SS-34, OTD-65, rotor: T-865.1 and AH-627, tubes: polycarbonate, no. 3137, polypropylene, no. 3138, polycarbonate, no. 03020, polyallomer, no. 03126 and no. 03141, bottle cap: no. 03613, Du Pont Instruments, D-6350 Bad Nauheim microvials: type "Eppendorf", polypropylene, no. 3810, Netheler und Hinz GmbH, D-2000 Hamburg 65
Chemicals:	sucrose, no. 7653, potassium bicarbonate, no. 4854, potassium dihydrogenphosphate, no. 4873, di-potassium hydrogenphosphate, no. 5099,

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EDTA, no. 8418,
E. Merck,
D-6100 Darmstadt 1

Procedure: killing of rat 5 days after Aroclor administration by cervical dislocation. Starvation 12 h before killing. All manipulations after dissection and removal of the liver performed between 0 and 5 degrees centigrade. Homogenization of 22.6 g rat liver using 45 ml 100 mmol potassium hydrogen phosphate/l and 10 mmol EDTA/l, pH 7.4, as homogenization buffer. Centrifugation of the crude homogenate at 1.08E4 m/s2 (= 1100 x g) for 2 min, and the resultant supernatant at 1.4E5 m/s2 (= 14500 x g) for 10 min. Addition of 120 ml 1.5 mol potassium hydrogen carbonate/l to the supernatant (approx. 60 ml), and centrifugation at 9.8E5 m/s2 (= 100000 x g) for 1 h. Resuspension of the pellet in 40 ml bidistilled water, 40 ml 100 mmol potassium hydrogen phosphate/l, pH 7.4, and re-centrifugation at 9.8E5 m/s2 (= 100000 x g) for 1 h. Resuspension of the pellet in 10 ml 100 mM Tris-HCl/l, pH 7.5, and gasing 50 ul aliquots with nitrogen. Freezing and storage at -75 degrees centigrade.

Scientific version: SOP BC 267/2
Text version: 11.Feb.87

5.2 Determination of Aryl Hydrocarbon Monooxygenase (EC 1.14.14.2) Activity

Principle: fluorometric determination of 3- and 9-hydroxy-B(a)P formed during the incubation of B(a)P with tissue extracts and separated after incubation by HPLC

Time

Sampling: -

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Determination:	within 1 day after preparation of microsomes
Sample material and quantity:	liver microsomes, 25 to 250 ug protein (linear assay response up to 250 ug protein (see FIGURE A))
Results expressed in:	U/mg protein (1 U = 1 nmol 3- plus 9-hydroxy-B(a)P/h)
Equipment:	high performance liquid chromatograph: model 1084 B, Hewlett-Packard GmbH, D-7500 Karlsruhe column: RP-18, length: 250 mm, inner diameter: 4.6 mm, particle diameter: 10 um, Dr. H. Knauer KG, D-6370 Oberursel incubation vessels: no. 611-52, Sovirel, La Vallois Perret, France injection vial: no. 70214, capacity: 1 ml, Machery-Nagel GmbH und Co. KG, D-5160 Düren spectrofluorometer: model 650-10S, Perkin Elmer GmbH, D-7770 Überlingen shaking water bath: no. 3047, Köttermann KG, D-3165 Hänigsen centrifuge: model J6, rotor: JS-4.2, Beckman Instruments GmbH, D-8000 München 4o
Chemicals:	magnesium chloride, no. 5833, acetonitrile, no. 30, sodium dihydrogen phosphate, no. 6346, acetone, no. 14, methanol, no. 6007, hydrochloric acid, no. 9057, saccharose, no. 7651, EDTA, no. 8418, E. Merck, D-6100 Darmstadt 1

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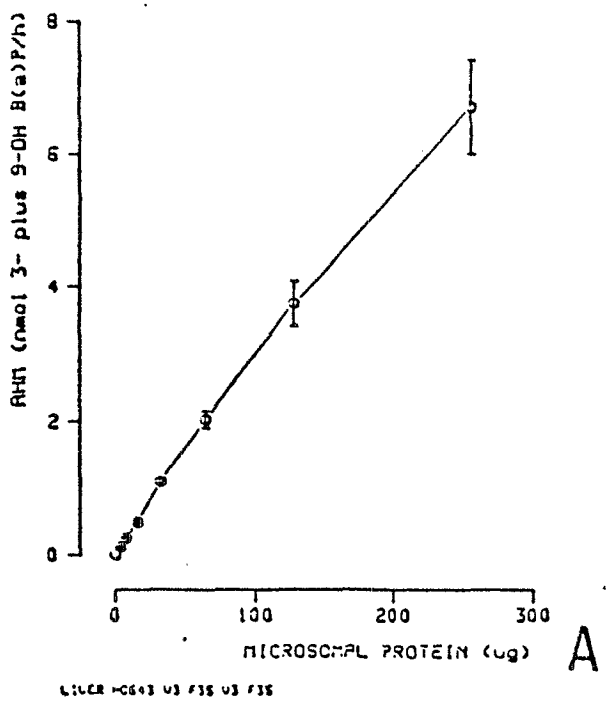
Source: https://www.industrydocuments.ucsf.edu/docs/yldl0000



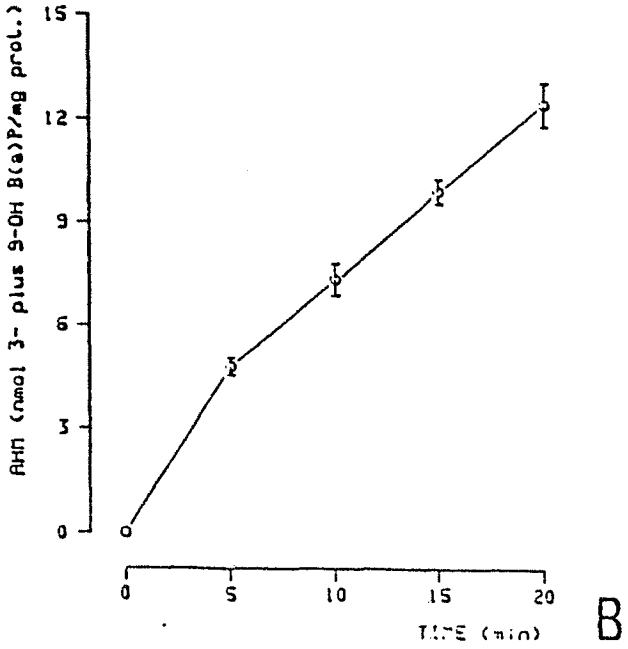


FIGURE A

DETERMINATION OF AHM ACTIVITY OF LIVER MICROSOMES, PROTEIN RESPONSE (A) AND TIME RESPONSE (B)

Remarks: the mean of triplicate determinations with sample errors,
A: incubation time: 10 min, B: 16 ug microsomal protein

date of determination: 22.Dec.81 and 14.Jan.82

scientific version: SOP BC 1/8 to 1/12

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Source: https://www.industrydocuments.ucsf.edu/docs/yldl0000

glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), no. 197726,
glucose-6-phosphate (G6P), no. 127027,
NADP, no. 128031,
NAD, no. 127981,
Boehringer Mannheim GmbH,
D-6800 Mannheim 31

benzo(a)pyrene (B(a)P), no. 3176,
Carl Roth GmbH,
D-7500 Karlsruhe 21

benzanthracene, no. B 220-9,
Ega Chemie,
D-7924 Steinheim

bovine serum albumin (BSA), no. A 4378,
tris(hydroxymethyl)aminomethane (Tris), no. T 1503,
Sigma Chemie GmbH,
D-8024 Deisenhofen

9-hydroxy-benzo(a)pyrene, no. 106,
3-hydroxy-benzo(a)pyrene, no. 75,
IIT Research Institute,
Chicago, Illinois 60616, USA

Tris buffer:
250 mmol/l, adjusted to pH 7.6 with hydrochloric acid

solution for termination of enzyme reaction:
methanol containing 0.15 mg/l benzanthracene (-20 degrees centigrade)

SVT buffer:
250 mmol saccharose/l, 5 mmol EDTA/l, 20 mmol/l Tris, pH 7.4, pH adjusted with hydrochloric acid

Procedure

Enzyme incubation: according to Van Cantfort et al. (1977), modified by INBIFO

triplicate determination per sample

incubation time: 10 min

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Source: https://www.industrydocuments.ucsf.edu/docs/yldl0000

	10 to 15 min: elution gradient from 700 ml aceto-nitrile/l to 1000 ml acetonitrile/l 15 to 20 min: elution gradient from 1000 ml acetonitrile/l to 400 ml acetonitrile/l regeneration time between 2 analyses: 3 min
Column pressure (MPa):	5 to 10
Detection:	fluorometric, excitation wavelength: 305 nm, emission wavelength: 430 nm, slit of excitation and emission monochromators: 15 nm
Calculation:	by HPLC microprocessor on the basis of external (3- plus 9-hydroxy-B(a)P) and internal (benzanthracene) standards
Scientific version:	SOP BC 1/12
Text version:	22.Jan.87

5.3 Determination of Protein Concentration (Biuret Method)

Principle:	photometric determination of a dye complex formed between peptide bonds and copper ions in alkaline solution
Time	
Sampling:	-
Determination:	-
Sample material and quantity:	microsomal suspension, 10 ul

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Source: https://www.industrydocuments.ucsf.edu/docs/yldl0000

Results expressed in:	g/l
Equipment:	ABA-100 Bichromatic Analyzer, Abbott GmbH, Diagnostics Division, D-6236 Eschborn
Chemicals:	test set total protein (Biuret method), no. 124281, standard proteins: Precimat, no. 125601, Precinorm U, no. 171735, Boehringer Mannheim GmbH, D-6800 Mannheim 31

Procedure

Reference:	Weichselbaum (1946), adapted by INBIFO to the bichromatic analyzer
Assay parameters:	each sample determined in duplicates

final concentration of components in assay mixture:

sodium hydroxide	0.1 mol/l
potassium sodium tartrate	15.7 mmol/l
potassium iodide	14.7 mmol/l
copper sulfate	5.9 mmol/l

photometric determination:
wavelength 1: 550 nm
wavelength 2: 650 nm

Storage until determination:	-75 degrees centigrade
Standard curve:	see FIGURE B
Detection limit:	1 g/l (empirical)
Reproducibility:	2.4 0/0 (mean RSD, N = 30, 1 to 60 g/l)

Scientific version:	SOP BC 128/2
Text version:	22.Jan.87

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Source: https://www.industrydocuments.ucsf.edu/docs/yldl0000

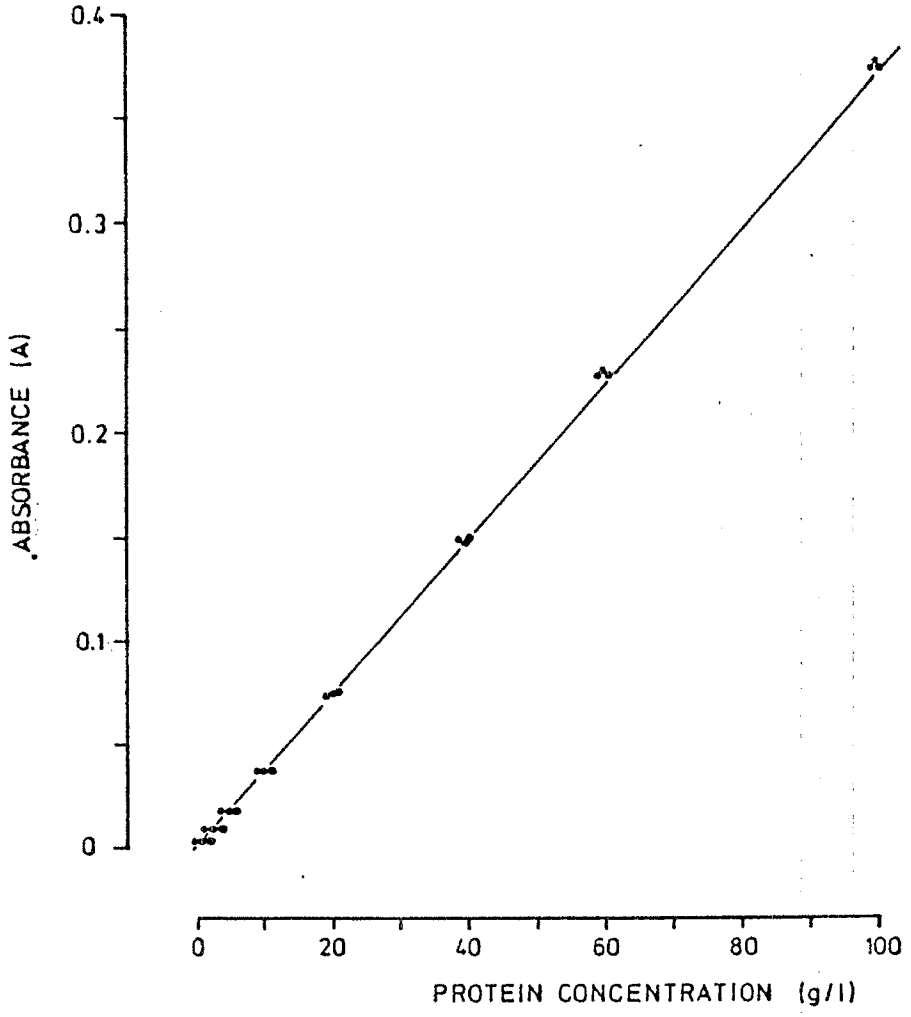

FIGURE B

PROTEIN STANDARD CURVE, BIURET METHOD

Remarks: SOP BC 128/2

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Source: https://www.industrydocuments.ucsf.edu/docs/yldl0000

5.4 Incubation of DNA with Test Substance

Principle:	incubation of test substance with DNA in vitro in the absence or presence of metabolizing systems (i. e. microsomes)
Time:	-
Sample material and quantity:	(1) 1 ug DNA (2) test substance in acetone or other adequate solvent
Results expressed in:	-
Equipment:	incubator: Techne Dri Block DB-3, Labora, Karl Heinz Bässler, D-6395 Weilrod-Winden centrifuge: Sigma 2MK, Sigma Laborzentrifugen GmbH, D-3360 Osterode microvials: type "Eppendorf", polypropylene, no. 3810, Netheler und Hinz GmbH, D-2000 Hamburg 65 freezer: no. 8218, Forma Scientific, via Labotect, D-3406 Bovenden-Göttingen
Chemicals:	DNA plasmid pBR322, no. 481238, DNA plasmid pUR222, no. 669776, nicotinamide adenine dinucleotide, reduced form (NADH), no. 107735, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), no. 621692, Boehringer Mannheim GmbH, D-6800 Mannheim 31 acetone, no. 14, EDTA, no. 8418,

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Source: https://www.industrydocuments.ucsf.edu/docs/yldl0000

hydrochloric acid, no. 9057,
E. Merck,
D-6100 Darmstadt 1

tris(hydroxymethyl)aminomethane
(Tris), no. T 1503,
Sigma Chemie GmbH,
D-8024 Deisenhofen

Procedure:

final concentration of components in assay mixture without metabolic activation:

DNA	20 mg/l
Tris-HCl, pH 8.0	10 mmol/l
EDTA	1 mmol/l
acetone (a)	.LE.50 ml/l

total assay volume: 100 ul

incubation time:
0.25 h to 2 h at 37 degrees centigrade

final concentration of components in assay mixture with metabolic activation:

microsomal protein	0.1 to	2.5	g/l
NADH	0.5 to	2	mmol/l
NADPH	0.5 to	2	mmol/l
Tris-HCl, pH 8.0		10	mmol/l
EDTA		5	mmol/l
acetone (a)		50	ml/l

total assay volume: 100 ul

incubation time:
0.25 h to 2 h at 37 degrees centigrade, removal of protein by phenol extraction, SOP BC 248/2

Scientific version: SOP BC 32/2
Text version: 22.Jan.87

(a) as solvent for test substance

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Source: https://www.industrydocuments.ucsf.edu/docs/yldl0000

5.5 Phenol Extraction

Principle:	removal of protein from aqueous solutions by extraction with phenol
Time:	immediately after sample incubation
Sample material and quantity:	incubation mixture of test substance with DNA and microsomes, with protein concentrations up to approx. 2.5 g/l, 100 ul
Results expressed in:	-
Equipment:	centrifuge: type "Eppendorf", no. 5412, test-tubes: type "Eppendorf", polypropylene, no. 3810, Netheler und Hinz GmbH, D-2000 Hamburg 65 whirlmix: Cenco Deutschland GmbH, D-5667 Haan
Chemicals:	sodium acetate, no. 8418, chloroform, no. 2445, ethanol, no. 983, hydrochloric acid, no. 9973, E. Merck, D-6100 Darmstadt 1 tRNA, no. 109517, Boehringer Mannheim, D-6800 Mannheim 31 tris(hydroxymethyl)aminomethane (Tris), no. T 1503, Sigma Chemie GmbH, D-8024 Deisenhofen phenol, no. 540-5509 VA, Gibco BRL GmbH, D-7514 Eggenstein

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Source: https://www.industrydocuments.ucsf.edu/docs/yldl0000

phenol solution:
80 ml phenol repeatedly equilibrated with 20 ml 0.01 mol/l Tris buffer pH 7.4 until neutral pH of the phenol and phenol saturated with water

sodium acetate solution:
3 mol/l (408.24 g/l), pH 6.0

tRNA solution:
1 g/l, in sterile bidistilled water

Tris buffer:
0.01 mol/l (1.2 g/l), adjusted to pH 7.4 with hydrochloric acid

ethanol (approx. 800 ml/l):
83 ml ethanol (960 g/l) and 17 ml bidistilled water

Procedure:

addition of 1 volume phenol solution to the sample, mixed 1 min on a whirlmix, centrifuged 2 min, upper (aqueous) phase transferred to a new test tube, repeated twice (for higher protein concentrations repeated several times, up to 6 times, to remove protein completely), aqueous phase extracted 2 to 3 times with 1 volume chloroform to eliminate traces of phenol, addition of tRNA to 100 mg/l, 1/10 volume sodium acetate solution and 3 volumes ethanol (9600 ml/l), 30 min at -75 degrees centigrade, centrifuged 10 min, supernatant discarded, pellet washed with 1 ml ethanol (800 ml/l) mixed briefly on a whirlmix, centrifuged 5 min, supernatant discarded, and pellet dried for a few minutes in a dessiccator

Scientific version: SOP BC 248/2
Text version: 22.Jan.87

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Source: https://www.industrydocuments.ucsf.edu/docs/yldl0000

5.6 Heat Posttreatment

Principle:	incubation of substance-treated DNA at high temperature, in order to generate nicks at sites where DNA lesions have been introduced by substance treatment
Time:	after 1st ethanol-precipitation of substance-treated DNA
Sample material and quantity:	substance-treated DNA dissolved in Tris-EDTA solution (40 ul)
Results expressed in:	-
Equipment:	Techne Dri Block DB-3, Labora, Karlheinz Bässler, D-6395 Weilrod-Winden microvials: type "Eppendorf", polypropylene, no. 3810, Netheler und Hinz GmbH, D-2000 Hamburg 65
Chemicals:	tris(hydroxymethyl)aminomethane (Tris), no. T 1503, Sigma Chemie GmbH, D-8024 Deisenhofen EDTA, no. 8418, E. Merck, D-6100 Darmstadt 1 TE buffer: 10 ml Tris/HCl buffer 1 mol/l, pH 8.0, 2 ml EDTA solution 0.5 mol/l, pH 8.0, 988 ml distilled water
Procedure:	dissolving of dried DNA pellet in 40 ul TE buffer, incubation at 70 degrees centigrade for 30 min, cooling to room temperature and preparing for gel electrophoresis
Scientific version:	SOP BC 282/1
Text version:	10.Feb.87

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Source: https://www.industrydocuments.ucsf.edu/docs/yldl0000